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Possible requirement of serum progression factors for transformation of BALB/c 3T3 fibroblasts by v-*Ras* p21

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To form colonies in soft agar, *ras*-transformed 3T3 fibroblasts require serum. We examined what growth factors in serum were essential for *ras*-induced transformation. Temperature-sensitive (*ts*) v-Ki-*ras*-transfected BALB/c 3T3 cells were used to strictly control both the activity of the *ras* protein and the cell cycle. When G_0-arrested *ts* cells were cultured with 10% serum at a permissive temperature, >50% of cells formed colonies. A similar colony-forming activity was observed in the presence of 10% platelet-poor plasma, but not in the presence of 10% plasma isolated from hypophysectomized rats. Inhibitors of IGF signals attenuated colony formation in the presence of serum. These data suggest that progression factors, probably IGFs, are essential components in serum for *ras*-induced transformation of 3T3 fibroblasts.

ras Action; Progression factor; Colony formation

1. INTRODUCTION

Ras oncoproteins are a family of low molecular weight guanine nucleotide-binding proteins [1]. Although other oncogenes, such as *myc*, are generally required to transform cells in addition to *ras* protein activity, activation of *ras* p21 is sufficient for cell transformation in 3T3 fibroblasts [2]. Thus, 3T3 fibroblasts may be a useful model in which to elucidate the role and function of *ras* proteins in transformation. Even in these cells, however, it is well known that serum is a requisite for *ras*-induced transformation. For instance, v-Ki-*ras*-transfected NIH 3T3 cells need 5–10% serum to form colonies in soft agar. This is also the case with BALB/c 3T3 cells. Nevertheless, which of the factors in serum that are essential for *ras*-induced transformation is still unclear. Addressing this question is expected to identify the intracellular systems that *ras* proteins seriously affect downstream.

In BALB/c 3T3 cells, the combination of PDGF (platelet-derived growth factor) and EGF (epidermal growth factor) allows IGF (insulin-like growth factor)-I or IGF-II to stimulate DNA synthesis [3]. Thus, there are at least two kinds of serum growth factors for these cells: competence factors (PDGF, EGF) and progression factors (IGF-I and -II). Based on these studies, we recently found [4] that activation of viral Ki-*ras* protein mimics the action of PDGF and EGF. This suggests that targets of the action of oncogenic *ras* proteins may be the IGF-I- and IGF-II-triggered pathways, prompting us to test the effect of IGF-deficient serum derived from hypophysectomized rats on *ras*-induced transformation. Since the action of growth factors may depend on the cell cycle, strict control of the cell cycle of individual cells was essential for the detailed analysis of growth factor actions. For this purpose, a temperature-sensitive Ki-*ras* BALB/c 3T3 cell (*ts* cell) line was found to be a useful tool [4], because both the cell cycle and the *ras* protein activity are controllable. The present results clearly indicate that *ras* protein requires the activity of progression factors, probably IGFs, to transform BALB/c 3T3 fibroblasts.

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2. EXPERIMENTAL

2.1. *Cells*

Temperature-sensitive Ki-*ras* BALB/c 3T3 cells (clone A31, subclone 714) (*ts* cells) and parental BALB/c 3T3 cells (clone A31), which were originally described in [5], were cultured and G_0-arrested as described [4]. These cells are permissive for viral *ras* activity at 37°C, and nonpermissive at 40°C. Temperature control was strictly regulated by means of media with an appropriate temperature and a CO_2 incubator.

2.2. *Colony-forming assay*

A colony-forming assay was performed in a 96-well dish as follows. A basal agar layer (50 μl/well) consisted of 25 μl MEM (Eagle's minimal essential medium) concentrated twice with serum or plasma, and 20 μl of 1.2% agar (final 0.5%) (Difco). G_0-arrested cells were suspended in MEM plus 0.5% agar, 50 μl of which was overlaid on the basal agar layer. On the solidified agar layers, 100 μl of a liquid

MEM containing the same concentration of serum or plasma as that in the basal layer was overlaid. The soft agar culture was carried out over 5 days at 37 or 40°C, and the number of colonies counted. The concentration of serum or plasma was expressed with that in the basal agar and the upper liquid layers. When the effect of pertussis toxin, tetramethrin, nitrendipine or cobalt was examined, these reagents were added to the upper liquid layer only. Their concentrations were thus expressed as those in the upper layer.

2.3. *Materials*

Normal PPP (platelet-poor plasma) was prepared as described [6]. PPP isolated from a 10-day-hypophysectomized rat, referred to as hypox PPP, was prepared in a similar manner. Two different batches of hypox PPPs were prepared individually from freshly frozen plasma of two hypophysectomized rats. The effect of hypophysectomy was verified by the finding that these rats suffered from diabetes inspidus after operation. These batches of freshly frozen plasma were generously provided by Toyo Jozo (Tokyo, Japan) and Shionogi (Osaka, Japan), respectively. The concentrations of IGF-I and IGF-II in these hypox PPPs were under the detection limit of radioimmunoassay for IGF-I and IGF-II (data not shown). Tetramethrin was kindly provided by Sumitomo Chemicals (Osaka, Japan). Pertussis toxin was purchased from Funakoshi (Japan).

3. RESULTS AND DISCUSSION

3.1. *Efficiency of colony formation of* ts *cells at 37 and 40°C*

When *ts* cells were cultured with 10% fetal calf serum (FCS) in soft agar at 40°C for 5 days, approximately 25% of the total cells formed colonies (110 ± 20 colonies/400 cells; n=9, means ± SE). In contrast, when cultured under the same conditions at 37°C, 56% of the cells formed colonies (223 ± 8 colonies/400 cells, n=9). Fig. 1 indicates the dose–response for the effect of serum at 37 and 40°C. In the presence of ≤ 1% serum, no colony formation was observed at either 37 or 40°C. In contrast, the colony-stimulating effect of serum at 37°C reached saturation at 5%. These results indicate that the transformation of *ts* cells required serum.

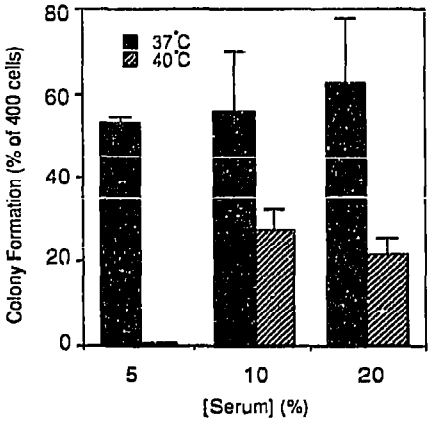

Fig. 1. Effect of serum on colony of *ts* cells. Temperature-sensitive Ki-*ras* BALB/c 3T3 cells (*ts* cells, 400 cells/well) were cultured in soft agar for 5 days in the presence of increasing concentrations of fetal calf serum (FCS) at permissive (37°C) or nonpermissive (40°C) temperatures, and the number of colonies counted. The colony formation was expressed as a percent of the total cell number. The data represent the mean ± SE of 9 experiments. No colonies were observed in the presence of 1% FCS at 37 and 40°C.

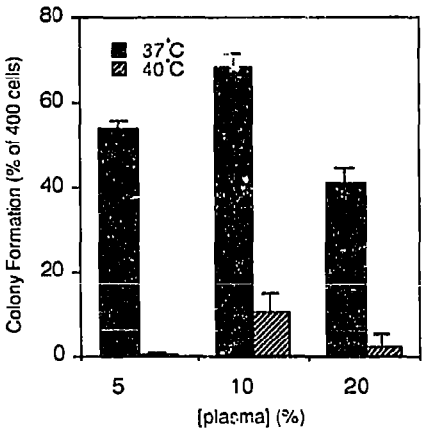

Fig. 2. Effect of platelet-poor plasma on colony formation of *ts* cells. The number of colonies was counted after the soft agar-culture of *ts* cells with various concentrations of PPP at 37 or 40°C under the same conditions as described in the legend to Fig. 1. The data represent the mean ± SE of 9 experiments. No colonies were observed in the presence of 1% PPP at either temperature.

3.2. *Effect of normal PPP on colony formation*

When *ts* cells were cultured with 10% PPP in soft agar at 37°C for 5 days, 68 ± 4% of the cells formed colonies (273 ± 13 colonies/400 cells, n=9, means ± SE). In contrast, when cultured under the same conditions at 40°C, only 10% of the total cells produced colonies (42 ± 6 colonies/400 cells, n=9). The dose–response for the effect of PPP at 37 and 40°C is very close to that of serum at either temperature (Fig. 2). In the presence of ≤ 1% PPP, no colony formation was observed at either 37 or 40°C. In contrast, the colony-forming effect of PPP at 37°C reached saturation at 5% to the level comparable to the effect of 5% serum. Since PPP is depleted with competence factors, these results indicate that competence factors in serum may be dispensable for *ras*-induced transformation of 3T3 fibroblasts.

3.3. *Effect of hypox PPP on colony formation*

It is well known that the serum components required for cell cycle progression of BALB/c 3T3 cells in late G_1 phase, referred to as progression factors, are IGF-I and -II [3]. To determine the requirement for IGFs in the action of PPP on *ras*-induced transformation, hypox PPP were used instead of PPP (Fig. 3). For this purpose, we used two batches of hypox PPP. In the presence of one batch of 10% hypox PPP, only 11 ± 7 colonies/400 cells (n=10, means ± SE) were observed at 37°C, whereas 10% PPP produced reproducible stimulation of colony formation under the same conditions (185 ± 7 colonies/400 cells, n=10). A different batch of hypox PPP yielded similar results (data not shown). These results suggest that pituitary-dependent plasma factors,

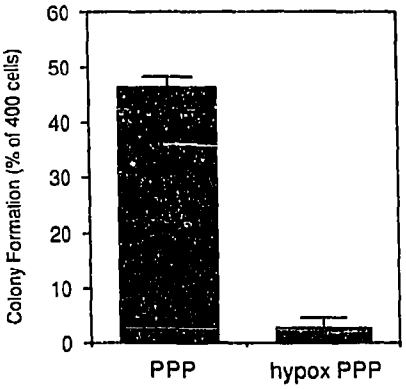

Fig. 3. Effect of hypox PPP on colony formation. Temperature-sensitive cells were cultured in soft agar in the presence of 10% PPP or hypox PPP (PPP prepared from a hypophysectomized rat) at 37°C for 5 days, and the colony numbers were counted. The data represent the mean ± SE of 10 experiments.

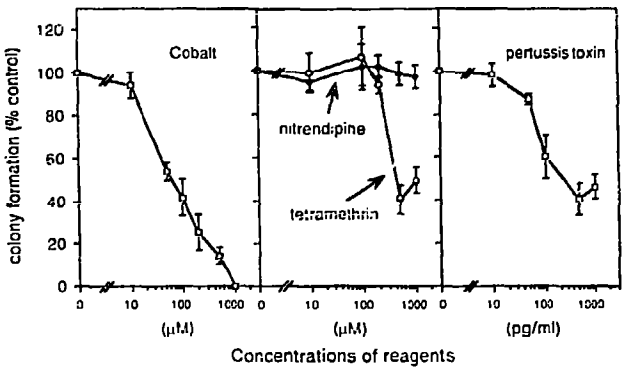

Fig. 4. Effect of IGF inhibitors on colony formation induced by serum in *ts* cells at permissive temperature. Cells were cultured at 37°C in the presence of 10% FCS and various concentrations of cobalt (⊡), tetramethrin (○), nitrendipine (●) or pertussis toxin (□) in soft agar for 5 days. The colony-forming activity was expressed as percent of the colony formation in the absence of reagents. The data represent the mean ± SE of 4 experiments.

probably IGFs, are serum components essential for *ras*-induced transformation of BALB/c 3T3 cells.

3.4. *Effect of inhibitors of IGF action on serum-induced colony formation*

In Balb/c 3T3 cells, cobalt and tetramethrin block IGF-I and IGF-II-induced calcium influx and calcium-permeable cation channel activation by IGF-I and IGF-II, possibly by acting on ion channels [7–9]. In contrast, pertussis toxin inhibits the actions of both IGF-I and IGF-II in these cells by acting on G_i proteins [7,10]. Thus, these reagents may utilize different mechanisms of action to inhibit IGF signals. To determine whether IGFs are essential factors in serum for *ras*-induced transformation of BALB/c 3T3 cells, we tested the effect of these IGF-inhibitors on *ras*-induced colony-formation of *ts* cells in the presence of 10% FCS. Pertussis toxin, cobalt, and tetramethrin inhibited the colony formation dose-dependently (Fig. 4). The IC_{50} values were 70 pg/ml for pertussis toxin, 30 μM for cobalt, and 300 μM for tetramethrin. In contrast, nitrendipine, which fails to affect IGF-induced calcium influx [7], had no significant effect on *ras*-induced transformation. It should be noted that treatment of cells with 1 mM cobalt abrogated *ras*-induced colony formation, whereas sufficient concentrations of pertussis toxin (1 ng/ml) or tetramethrin (3 mM) inhibited colony formation by only 50%. Although it is not yet clear whether these reagents specifically act on IGF signals, neither cobalt, pertussis toxin nor tetramethrin inhibits polyphosphoinositide breakdown induced by PDGF in BALB/c 3T3 cells (data not shown), suggesting that their action of IGF signals is rather specific. These results support the idea that the mitogenic intracellular pathways of IGFs are one of the targets of the transforming action of *ras* proteins.

It remains unclear why the inhibition of colony formation by pertussis toxin and tetramethrin was partial, while cobalt completely inhibited *ras*-induced transformation. However, it has been reported [7] that pertussis toxin and tetramethrin reduce IGF-induced calcium influx to the level comparable to the basal influx, whereas cobalt completely abolishes calcium influx. Thus, it is conceivable that *ras* function, plus the basal level of calcium influx, may allow a considerable portion, but not all the population of the cells to transform, and IGF-increased calcium influx may be requisite for the *ras*-induced transformation of the remainder of the cells.

4. CONCLUSION

We report here that progression factors, possibly IGF-I and/or -II, are required for serum to enable *ras* proteins to transform BALB/c 3T3 cells. Obviously, we should await the results of further experiments using purified IGFs in the presence or absence of hypox PPP. Furthermore, it should be determined whether this is also the case in transformed 3T3 cells with constitutively active *ras* proteins or in other types of cells. However, the present study indicates that *ras* proteins may accomplish their transforming function by acting on the functions of IGFs, suggesting that at least one downstream target of *ras* protein action is the signaling pathway triggered by IGFs. This idea is consistent with our previous report [4] showing that activation of *ras* proteins in BALB/c 3T3 cells permits the IGF-II receptor to couple to oligomeric GTP-binding proteins. Combined with the fact that competence factors affect the actions of IGFs [3], this idea also occurs with the recent report showing that competence factors phosphorylate GAP (GTPase activating protein) and thereby affect the activity of *ras* proteins in 3T3 fibroblasts [11]. Finally, this

study opens the possibility of controlling *ras*-mediated transformation by modulating the activity of IGF signals.

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